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Letter

Stress affects the epigenetic marks added by *Bari-Jheh:* a natural insertion associated with two adaptive phenotypes in Drosophila

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ABSTRACT

Transposable elements are emerging as an important source of cis-acting regulatory sequences and epigenetic marks that could influence gene expression. However, few studies have dissected the role of specific transposable element insertions on gene regulation. *Bari-Jheh* is a natural transposon that mediates resistance to oxidative stress by adding cis-regulatory sequences. In this work, we integrated publicly available data with chromatin immunoprecipitation and immune response assays to get a more comprehensive picture of *Bari-Jheh* molecular and functional effects. We showed that *Bari-Jheh* is associated with H3K27me3 enrichment, which is consistent with expression changes in adjacent genes. We further showed that stress affects the histone marks introduced by *Bari-Jheh*, which correlates with further expression changes. Finally, we found that flies with *Bari-Jheh*, which are resistant to oxidative stress, are also more tolerant to bacterial infection. We conclude that *Bari-Jheh* influences gene expression and enables stress response through two different mechanisms, by adding cis-regulatory sequences and by adding histone marks, leading to changes in two ecologically relevant phenotypes.

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INTRODUCTION

Gene regulation is a complex process that involves mechanisms at the DNA sequence level and at the epigenetic level. Although genes can acquire novel regulatory mechanisms through different types of mutations, transposable elements (TEs) are emerging as an important source of regulatory variation (Slotkin and Martienssen 2007; Cowley and Oakey 2013). TEs can contain cis-regulatory sequences that affect the expression of nearby genes. Some of the recent examples on the global impact of TEs on gene expression levels include: providing enhancer sequences that contribute to the stress-induced gene activation in maize, adding transcription factor binding sites in the mouse and the human genomes, and providing alternative transcription start sites in Drosophila (Batut, et al. 2013; Sundaram, et al. 2014; Makarevitch, et al. 2015). The epigenetic status of TEs can also affect gene regulation. In *Arabidopsis thaliana*, gene transcription is affected by the methylation status of intragenic TEs (Le, et al. 2015) and correlates with siRNA-targeting of TEs (Wang, et al. 2013). In Drosophila, local spreading of repressive heterochromatin marks from TEs has been associated with gene downregulation (Sentmanat and Elgin 2012; Lee 2015). Although all these studies strongly suggest that TEs may play a role in gene regulation through different molecular mechanisms, detailed analyses that link changes in expression with fitness effects are needed to conclude that TEs have a functional impact on gene expression.

There are a few examples in which TE-induced changes in gene expression have been shown to be functionally relevant (McCue, et al. 2012; Guio, et al. 2014; Mateo, et al. 2014). One of these cases is *Bari-Jheh*, a *Drosophila melanogaster* full-length transposon providing a cis-regulatory sequence that affects the expression of its nearby genes (Gonzalez, et al. 2008; Gonzalez, et al. 2009). *Bari-Jheh* is associated with downregulation of *Jheh2* and *Jheh3* in nonstress conditions, and with upregulation of *Jheh1* and *Jheh2* and downregulation of *Jheh3* under oxidative stress conditions (Guio, et al. 2014). We have previously shown that *Bari-Jheh* adds Antioxidant Response Elements to the upstream region of *Jheh2* leading to *Jheh2* upregulation under oxidative stress conditions (Guio, et al. 2014; Guio and Gonzalez 2015). How *Bari-Jheh* affects gene expression under nonstress conditions, and how *Bari-Jheh* affects *Jheh3* expression under oxidative stress conditions remains unexplored. In this work, we hypothesized that *Bari-Jheh* could also be affecting the expression of nearby genes by remodeling the local chromatin state. Moreover, because oxidative stress is also caused

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by gram-negative bacterial infection, we tested whether flies with *Bari-Jheh* are also more tolerant to bacterial infection (Lemaitre and Hoffmann 2007; Bou Sleiman, et al. 2015).

RESULTS

Bari-Jheh could be affecting the local chromatin state

To test whether *Bari-Jheh* could be affecting the local chromatin state, we analyzed the transposon sequence and its flanking regions (Figure 1). We first looked for Trithorax group Response Elements (TREs) that recruit H3K4 methyltransferases, and Polycomb group Response Elements (PREs) that recruit H3K27 methyltransferases (see Material and Methods) (Greer and Shi 2012; Schwartz and Pirrotta 2013). While H3K4me3 is associated with active chromatin, H3K27me3 is associated with facultative heterochromatin. We found no TREs in the sequence analyzed, but we found one PRE in the *Bari-Jheh* sequence and one PRE in the coding region of *Jheh3*, where modENCODE reports a Polycomb mediated repressive chromatin state (Figure 1A and 1B) (Ringrose, et al. 2003, Roy et al 2010; Schwartz and Pirrotta 2013).

To further test whether *Bari-Jheh* affects the local heterochromatin state, we also investigated whether *Bari-Jheh* has piRNA binding sites and/or recruits HP1a (see Material and Methods). Sites with homology to piRNAs behave as cis-acting targets for heterochromatin assembly, which is associated with HP1a and H3K9me2/3 (Sentmanat and Elgin 2012). We found that *Bari-Jheh* has sites with homology to piRNAs (Figure 1C). Accordingly, we also found that HP1a specifically binds to the *Bari-Jheh* sequence (Fig. 1D).

Thus, *Bari-Jheh* could be introducing PREs that would be involved in the recruitment of H3K27me3. Additionally, *Bari-Jheh* could also be inducing pi-RNA mediated heterochromatin assembly. These results provide suggestive but not conclusive evidence that *Bari-Jheh* could be contributing to the heterochromatic state of these region.

Bari-Jheh adds the heterochromatin mark H3K27me3 in nonstress conditions

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To experimentally test whether *Bari-Jheh* affects histone marks enrichment, we performed Chromatin Immune Precipitation (ChIP)-qPCR experiments in guts of adult flies with H3K4me3, H3K9me3 and H3K27me3 antibodies (supplementary Figure S1) (see Material and Methods). We compared the histone mark enrichment in both sides of *Bari-Jheh* insertion, *Bari-Jheh2* and *Bari-Jheh3* regions, with the corresponding region in flies without *Bari-Jheh*, *Bari*-Absent region (Figure 1A).
We found no significant differences in H3K4me3 or H3K9me3 enrichment between the strain with and without *Bari-Jheh* (Table 1 and Figure 2A). For H3K27me3, we found significant differences for the *Bari-Jheh3* region but not for the *Bari-Jheh2* region (Table 1 and Figure 2A). Thus, *Bari-Jheh* is associated with the facultative heterochromatin mark H3K27me3 in the *Bari-Jheh3* region.

Bari-Jheh adds the heterochromatin mark H3K9me3 in oxidative stress conditions

To further test whether oxidative stress affects the heterochromatin marks added by *Bari-Jheh*, we performed ChIP-qPCR experiments in flies exposed to paraquat (see Material Methods). We found no significant differences for H3K4me3 between flies with and without *Bari-Jheh* (Table 1 and Figure 2B). H3K9me3 was enriched in *Bari-Jheh2* and *Bari-Jheh3* regions compared with the *Bari*-Absent region (Table 1 and Figure 2B). Finally, H3K27me3 is only enriched in the *Bari-Jheh3* (Table 1 and Figure 2B).
Overall, these results showed that under oxidative stress conditions *Bari-Jheh* is associated with the constitutive heterochromatin mark H3K9me3 on both sides of the insertion and the facultative heterochromatin mark H3K27me3 only in the *Bari-Jheh3* region.

Flies with _Bari-Jheh_ are associated with increased oral infection tolerance

To test whether flies with *Bari-Jheh* were more gut immunocompetent than flies without *Bari-Jheh*, we performed an oral infection experiment using *Pseudomonas entomophila* (Vodovar, et al. 2005). We used flies with three different genetic backgrounds (see Material and Methods). In outbred populations #1 and outbred populations #2, we found that both female and male flies with the insertion were more tolerant to *P. entomophila* infection than flies without the insertion (Figure 3A and 3B,

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respectively, and Table 2). However, in introgressed strains we found no significant differences in females, while males with *Bari-Jheh* were more sensitive to oral infection than males without the insertion (Figure 3C and Table 2). Thus, *Bari-Jheh* is associated with tolerance to oral infection with *P. entomophila* in two different outbred populations but not in introgressed strains.

DISCUSSION

In the present work, we combined different sources of information to analyze whether *Bari-Jheh* insertion could be affecting the local chromatin state. We found evidence suggesting that *Bari-Jheh* could be associated both with H3K27 and H3K9 chromatin marks (Figure 1). We tested these predictions by performing ChIP experiments in adult flies, and we found that in nonstress conditions *Bari-Jheh* is associated with H3K27me3 histone mark enrichment (Figure 2A). Previous analyses have shown that different TE families are associated with specific histone marks (Rebollo, et al. 2012, Pezic et al 2014). Enrichment for H3K27me3 has previously been reported for the *roo* family, while other families were enriched both for H3K27me3 and H3K9me2 (Fablet, et al. 2009; Rebollo, et al. 2012; Akkouche, et al. 2013). The enrichment for heterochromatin histone marks is one of the epigenetic silencing mechanisms used to control the activity of TEs (Levin and Moran 2011; Gonzalez and Petrov 2012). However, heterochromatin formation triggered by TEs can also spread into the nearby genes affecting their expression (Sentmanat and Elgin 2012; Lee 2015). Accordingly, we found that *Bari-Jheh* that is associated with H3K27me3 facultative heterochromatin mark is also associated with downregulation of the expression of *Jheh2* and *Jheh3* genes in nonstress conditions (Gonzalez, et al. 2009; Guio, et al. 2014).

Oxidative stress has been associated with increases in several histone methylation marks (Niu, et al. 2015). Consistent with these results, we found that under oxidative stress conditions, besides H3K27me3 enrichment in the *Bari-Jheh3* region, *Bari-Jheh* insertion is associated with H3K9me3 enrichment both in *Bari-Jheh2* and in *Bari-Jheh3* regions (Figure 2B). Although H3K9me3 is often associated with gene silencing, there

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is evidence suggesting that this histone mark is also associated with gene activation (Vakoc, et al. 2005; Kouzarides 2007; Pezic et al 2014). Additionally, we have previously shown that *Bari-Jheh* adds Antioxidant Response Elements (AREs), which are necessary and sufficient to induce the upregulation of downstream genes (Sykiotis and Bohmann 2008; Chatterjee and Bohmann 2012; Guio, et al. 2014). Thus, *Bari-Jheh* could be affecting *Jheh2* expression under oxidative stress conditions both because it adds AREs and because it affects the local chromatin state. Our results also provide a mechanistic explanation for the downregulation of *Jheh3*: *Bari-Jheh* is associated with enrichment of both H3K9me3 and H3K27me3 in the 3' end of this gene. Combined histone marks can have different roles compared with the same histone marks appearing in isolation (Greer and Shi 2012; Lelli, et al. 2012). Additionally, the effects of histone modifications also depend on the relative position of the histone mark regarding the functional sequence (Vakoc, et al. 2005; Kouzarides 2007; Greer and Shi 2012). Thus it is possible that the combination of these two histone marks in the 3' region of *Jheh3* would lead to downregulation of this gene (Greer and Shi 2012, Lille et al 2012).

Besides elucidating that *Bari-Jheh* could also be affecting gene expression by changing the local chromatin state, we showed that *Bari-Jheh* is associated with increased tolerance to *P. entomophila* infection in two of the three backgrounds analyzed (Figure 3 and Table 1). These results are consistent with previous analysis showing that inbred strains tolerant to *P. entomophila* infection are more resistant to paraquat while inbred strains susceptible to *P. entomophila* could be resistant to paraquat (Bou Sleiman, et al. 2015). Variation in tolerance/susceptibility phenotypes among genetic backgrounds likely reflects the complex nature of immune response, which results from the interplay of many biological processes and it is highly dependent on environmental conditions (Lemaitre and Hoffmann 2007; Lazzaro and Little 2009).

Overall, our results provide further evidence for the complex effects of natural TE insertions on gene regulation and organismal phenotypes. A single mutation, influences gene expression through two different regulatory mechanisms and has fitness consequences on two relevant phenoypes: oxidative stress and immune response.

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MATERIAL AND METHODS

Fly stocks. We used the outbred populations and introgressed strains described in Guio et al (2014) and a new outbred population created for this work (Supplementary Table S1). All flies were kept in large embryo collection chambers as described in Guio et al (2014).

Oxidative stress exposure. To induce oxidative stress, we added paraquat to the fly food up to a final concentration of 10 mM. For nonstress conditions, we used regular food. We did three to six replicas, of 50 females each, for each condition and genotype.

Chromatin Immunoprecipitation assays

We performed ChIP assays in guts because the gut is the first barrier against oxidative stress. Guts of 5-day-old females were dissected in 1x PBS with protease inhibitor cocktail. Chromatin immunoprecipitation was performed as described in Silva-Sousa, et al. (2012) with the following changes: guts were resuspended in 2ml buffer A with 1,8% formaldehyde, and sonication was performed with 15 cycles of 30 seconds ON, 30 seconds OFF. All the solutions were made according to the instructions of *Magna ChIP G Kit* 17-611 from Millipore. Chromatin was immunoprecipitated (IP) with antibodies against H3K4me3 (Catalog # ab8580), H3K9me3 (#ab8898) and H3K27me3 (#ab6002). All the antibodies were ChIP grade and antibody quality was tested before performing the experiments (supplementary Figure S1). We did 3 to 6 biological replicas for each IP. We quantify the IP enrichment by qRT-PCR normalizing the data using the "input" of each IP as the reference value (ΔCt method, supplementary Table S2). Data was normalized using log transformation before performing ANOVA.

Prediction of PREs and TREs. We used the database JASPAR (Mathelier, et al. 2015) with 95% threshold to predict the presence of PREs/TREs in the region analyzed: chromosome 2R: 18856800-18861999 (dos Santos, et al. 2015).

Detection of piRNA reads. To search for piRNA homology sites in *Bari-Jheh*, we used reads from available piRNA libraries (Li, et al. 2009; Satyaki, et al. 2014; Shpiz et al 2014) and we followed the methodology described in Ullastres, et al. (2015).

Detection of HP1a binding sites. To analyze the binding sited for HP1a in the *Bari-Jheh* region we used HP1a modENCODE ChIP-Seq data (Kharchenko, et al. 2011) and we followed the methodology described in Ullastres et al (2015).

Oral infection assays. We used *Pseudomonas entomophila* to perform oral infection assays (courtesy of Dr. Bruno Lemaitre laboratory) following Neyen, et al. (2014)

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protocol. We used 10 vials per sex, per strain, and per condition. Before infection, the flies were starved during two hours. The size of the effect (odds-ratio) was measured when the weaker strain arrived at 50% mortality.

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FIGURE LEGENDS

Figure 1. ***Bari-Jheh* could be adding heterochromatin marks to the *Jheh* intergenic region.**

A) Schematic representation of *Jheh* genes in flies without *Bari-Jheh* and flies with *Bari-Jheh*. Black boxes represent exons, black arrows represent the direction of transcription, white boxes the 5'-UTR and 3'-UTR regions, the black line indicates intergenic or intronic regions and the red box represents *Bari-Jheh*. Orange lines represent the amplicons of the three regions analyzed using ChIP-qPCR experiments. The blue lines indicate the approximated position of the predicted PREs. B) modENCODE chromatin states in S2 cells and BG3 cells in the region analyzed. S2 cells and BG3 cells are derived from late male embryonic tissues and the central nervous system of male third instar larvae, respectively (Roy et al 2010). Colours and numbers represent different chromatin states. Note that although *Bari-Jheh* appears to be associated with state 30, modENCODE does not analyzed repetitive regions. The vertical discontinuous lines indicate the location of *Bari-Jheh* insertion. C) Mapping of piRNA reads in the *Bari-Jheh* and 1.5 kb flanking region. Reads mapping in sense orientation are represented in blue, and reads mapping in antisense orientation in red. D) Mapping of HP1a reads in the *Bari-Jheh* and 1.5 kb flanking regions. Reads from embryo stage are represented in blue, reads from larva L3 stage in green and reads from adult head in red.

Figure 2. Histone mark enrichment in *Bari-absent, Bari-Jheh2* and *Bari-Jheh3* regions.

Enrichment of the histone marks relative to the input of each strain, in A) nonstress conditions and B) oxidative stress conditions. Levels of H3k4me3, H3K9me3 and H3K27me3 in the *Bari-absent* (green), *Bari-Jheh2* (blue) and *Bari-Jheh3* (red) analyzed regions. Bars give the mean of three to six biological replicas (± SEM). Significant differences between regions are mark with an asterisk (p-value<0.05).

Figure 3. **Survival curves after *P. entomophila* infection in female and male flies with and without *Bari-Jheh*.**

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A) Survival curves for outbred populations #1, B) Survival curves for outbred populations #2, and C) Survival curves for introgressed strains. Survival curves in nonstress conditions are represented as discontinuous lines and survival curves after *P. entomophila* infection are represented as continuous lines. Survival of *Bari-Jheh* flies are represented in red and survival of flies without *Bari-Jheh* are represented in black. Each point represents the mean survival of 10 replicas and the error bars represent the standard error of the mean.

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Table 1. Statistical analyses of histone mark enrichment. Significant values are highlighted in bold.

Condition	**Regions compared**	**Mann-Whitney U-test p-value**		
		H3K4me3	**H3K9me3**	**H3K27me3**
Nonstress	*Bari-Jheh2 vs Bari-Absent*	0.827	0.827	0.127
	Bari-Jeh3 vs Bari-Absent	0.957	0.927	**0.0495**
Oxidative stress	*Bari-Jheh2 vs Bari-Absent*	0.286	**0.033**	0.088
	Bari-Jheh3 vs Bari-Absent	0.507	**0.031**	**0.033**

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Table 2. Statistical analyses of the *P .entomophila* infection survival experiments.

Genetic background	**Compared Strains**	**Sex**	**Logrank test p-value**	**Odds-ratio (confidence interval)**
Outbred #1	*Bari-Jheh* (–) *vs Bari-Jheh* (+)	Females	**<<0.0001**	2.03 (1.36 – 3.02)
	Bari-Jheh (–) *vs Bari-Jheh* (+)	Males	**<<0.0001**	19.46 (10.38 – 36.47)
Outbred #2	*Bari-Jheh* (–) *vs Bari-Jheh* (+)	Females	**0.028**	1.59 (1.07 – 2.36)
	Bari-Jheh (–) *vs Bari-Jheh* (+)	Males	**$3.6x10^{-4}$**	1.91 (1.28 – 2.84)
Introgressed	*Bari-Jheh* (–) *vs Bari-Jheh* (+)	Females	**0.262**	--
	Bari-Jheh (–) *vs Bari-Jheh* (+)	Males	**0.044**	3.23 (2.12 – 4.92)

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Figure 1

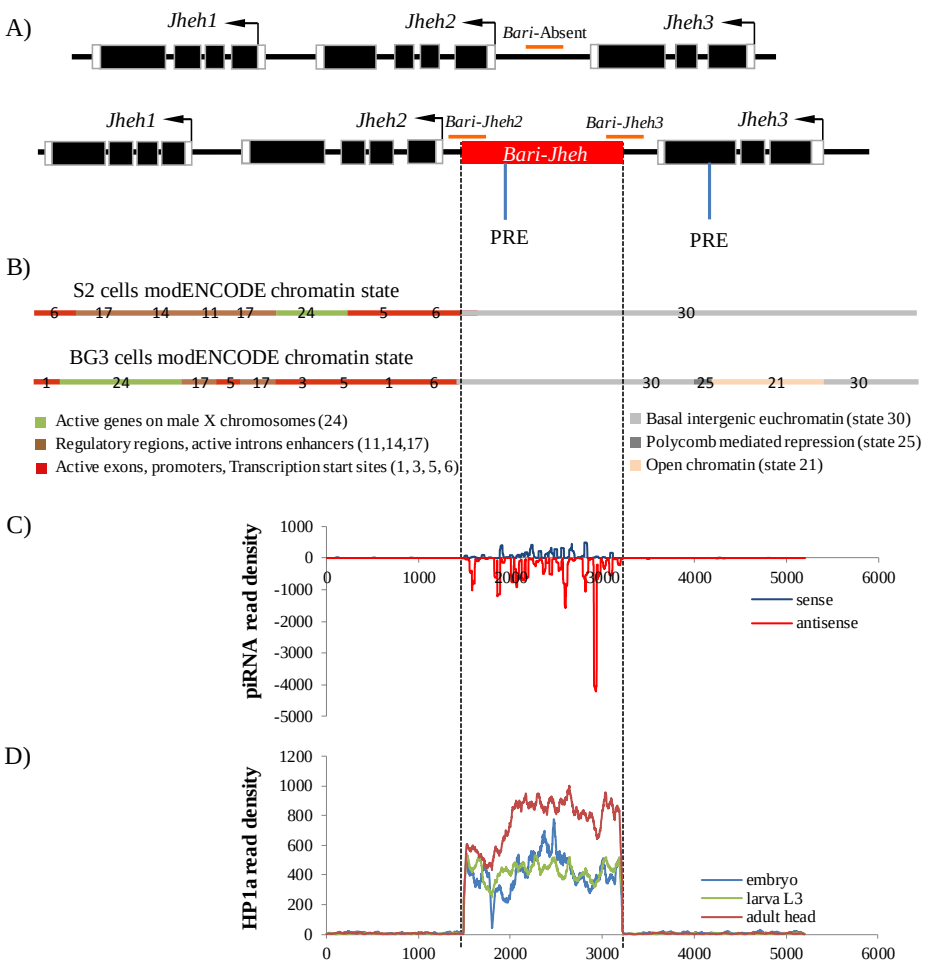

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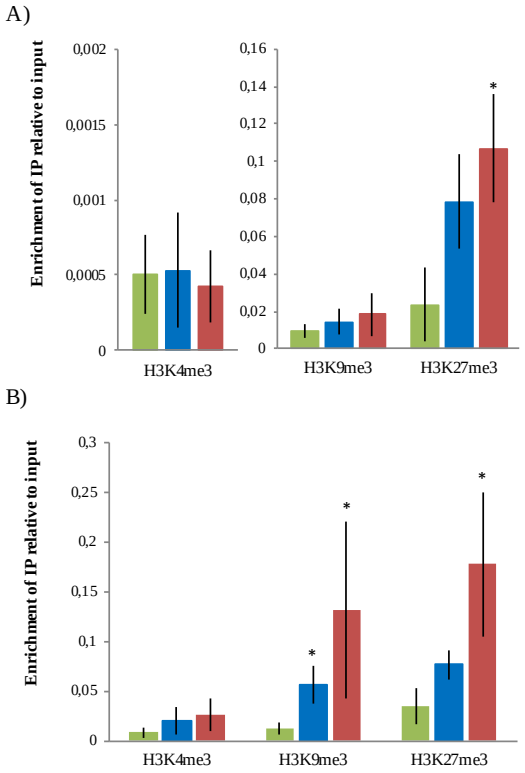
Figure 2



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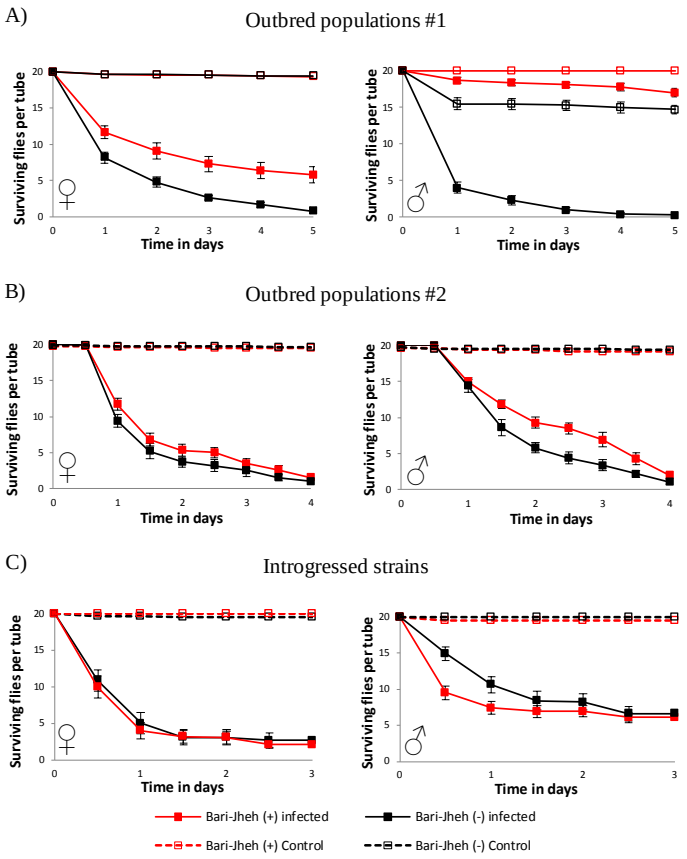
Figure 3